

METHODS FOR DIAGNOSIS AND PROGNOSIS OF CANCER

RELATED APPLICATIONS

[001] This application claims the benefit of U.S. Provisional Application No. 60/438,861, filed January 9, 2003.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

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BACKGROUND OF THE INVENTION

[003] Cancer remains a major health concern. Despite increased understanding of many aspects of cancer, the methods available for its treatment continue to have limited success. First of all, the number of cancer therapies is limited, and none provides an absolute guarantee of success. Second, there are many types of malignancies, and the success of a particular therapy for treating one type of cancer does not mean that it will be broadly applicable to other types. Third, many cancer treatments are associated with toxic side effects. Most treatments rely on an approach that involves killing off rapidly growing cells; however, these treatments are not specific to cancer cells and can adversely affect any dividing healthy cells. Fourth, assessing molecular changes associated with cancerous cells remains difficult. Given these limitations in the current arsenal of anti-cancer treatments, how can the best therapy for a given patient be designed? The ability to detect a malignancy as early as possible, and assess its severity, is extremely helpful in designing an effective therapeutic approach. Thus, methods for detecting the presence of malignant cells and understanding their disease state are desirable, and will contribute to our ability to tailor cancer treatment to a patient's disease.

[004] While different forms of cancer have different properties, one factor which many cancers share is the ability to metastasize. Until such time as metastasis occurs, a tumor, although it may be malignant, is confined to one area of the body.

This may cause discomfort and/or pain, or even lead to more serious problems including death, but if it can be located, it may be surgically removed and, if done with adequate care, be treatable. However, once metastasis sets in, cancerous cells have invaded the body and while surgical resection may remove the parent tumor, this does not address other tumors. Only chemotherapy, or some particular form of targeting therapy, then stands any chance of success.

[005] The process of tumor metastasis is a multistage event involving local invasion and destruction of the intracellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site(s), and growth in the new location(s) (Fidler, et al., *Adv. Cancer Res.* 28, 149-250 (1978), Liotta, et al., *Cancer Treatment Res.* 40, 223-238 (1988), Nicolson, *Biochim. Biophys. Acta* 948, 175-224 (1988) and Zetter, N. *Eng. J. Med.* 322, 605-612 (1990)). Success in establishing metastatic deposits requires tumor cells to be able to accomplish these steps sequentially. Common to many steps of the metastatic process is a requirement for motility. The enhanced movement of malignant tumor cells is a major contributor to the progression of the disease toward metastasis. Increased cell motility has been associated with enhanced metastatic potential in animal as well as human tumors (Hosaka, et al., *Gann* 69, 273-276 (1978) and Haemmerlin, et al., *Int. J. Cancer* 27, 603-610 (1981)).

[006] Tumor angiogenesis is essential for both primary tumor expansion and metastatic tumor spread (Blood et al., *Biochim. Biophys. Acta* 1032:89-118 (1990)). Angiogenesis is a fundamental process by which new blood vessels are formed. Progressive tumor growth necessitates the continuous induction of new capillary blood vessels which converge upon the tumor. In addition, the presence of blood vessels within a tumor provides a ready route for malignant cells to enter the blood stream and initiate metastasis. Thus, malignancy is a systemic disease in which interactions between the neoplastic cells and their environment play a crucial role during evolution of the pathological process (Fidler, I. J., *Cancer Metastasis Rev.* 5:29-49 (1986)).

[007] Identifying factors that are associated with tumor progression, particularly metastasis and angiogenesis, is clearly a prerequisite not only for a full understanding of cancer, but also for the development of rational new anti-cancer therapies. In addition to using such factors for diagnosis and prognosis, these factors represent important targets for identifying novel anti-cancer compounds, and are useful for

identifying new modes of treatment, such as inhibition of metastasis. One difficulty, however, is that the genes characteristic of cancerous cells are very often host genes being abnormally expressed. For example, a protein marker for a given cancer, while expressed in high levels in connection with that cancer, may also be expressed elsewhere throughout the body, albeit at reduced levels. Thus, some care is required in determining whether the expression of any single gene in a given cancer is a meaningful marker for the progression of the disease.

[008] Prostatic carcinoma is the most prevalent form of cancer in males and the second leading cause of cancer death among older males (Boring, et al., *Cancer J. Clinicians*, 7-26 (1994)). Clinically, radical prostatectomy offers a patient with locally contained disease an excellent chance for cure. If diagnosed after metastases are established, however, prostate cancer is a fatal disease, for which there is no effective treatment that significantly increases survival. The recent development of the prostate specific antigen (PSA) test has dramatically improved diagnosis, allowing earlier detection of prostate cancer and thus earlier treatment (Catalona, et al., *J. Urol.*, 151, 1283-1290 (1994)). Unfortunately, the PSA test does not predict which tumors may progress to the metastatic stage (Cookson, et al., *J. Urology* 154, 1070-1073 (1995) and Aspinall, et al., *J. Urology* 154, 622-628 (1995)). In addition, up to 75% of men who test positive for serum PSA do not have prostate cancer (Caplan & Kratz, *Am. J. Clin. Pathol.*, 117:S104-108 (2002); and Woolf, *Int. J. Technol. Assess Health Care*, 17(3):275-304 (2001). Such false positives lead to unenecessary medical procedures, and needless anxiety for a large number of men each year. Thus, there is a need in the art for additional biomarkers which can, alone or in combination with PSA or other biomarkers, increase the specificity and sensitivity of prostate cancer diagnosis. Additionally, the treatment and diagnosis of a variety of cancers would be significantly improved by methods for earlier detection, as well as by methods to assess the severity and metastatic potential of an individual's cancer.

SUMMARY OF THE INVENTION

[009] We have discovered a protein in humans, herein referred to as thymosin β 16 (SEQ ID NO:1), that is expressed in human prostate cancer tumors but not in specimens of benign prostate hyperplasia (BPH) tissues. In contrast, prostate specific antigen (PSA), the gold standard of prostate cancer diagnosis, is highly expressed in BPH tissues.

[0010] These results indicate that increased expression of thymosin β 16 has a high correlation to disease state in cancers including prostate cancer, lung carcinoma, breast carcinoma, thyroid carcinoma, brain cancers other than neuroblastoma (cerebellum, medulloblastoma, astrocytoma, ependymoma, glioblastoma), pancreatic carcinoma, ovarian carcinoma, uterine cancer, eye cancer (retinoblastoma), muscle (rhabdomyosarcoma), lymphoma, stomach cancer, liver cancer, colon cancer, kidney cancer, and is particularly associated with metastatic cancers. Preferably the cancer is of epithelial origin. As used herein the cancer is a cancer other than neuroblastoma. Preferably cancers other than brain cancer. Accordingly, assaying for enhanced levels of transcript or gene product can be used not only in a diagnostic manner, but also in a prognostic manner for particular cancers. Additionally, thymosin β 16 can be used alone or in conjunction with other cancer markers, e.g., PSA and thymosin β 15, in the diagnosis and prognosis of cancer. For example, PSA is a widely used diagnostic for prostate cancer, however detection of PSA leads to many false positives as well as false negatives. Monitoring the presence of thymosin β 16 along with levels of PSA increases the specificity and sensitivity of diagnosis of prostate cancer. In addition, as levels of thymosin β 16 are elevated in a variety of cancers, using methods for detection of thymosin β 16 in conjunction with specific cancer biomarkers increases the specificity and sensitivity of diagnosis using the specific markers.

[0011] The present invention provides a method of diagnosing cancer in a patient, especially cancers of epithelial origin, such as prostate cancer. The method comprises measuring the level of thymosin β 16 in a biological test sample obtained from the patient and comparing the observed level of thymosin β 16 with the level of thymosin β 16 present in a normal control sample of the same type. Higher levels of thymosin β 16 in the test sample, as compared to the normal control sample, is indicative of cancer.

[0012] The term "normal control sample" refers to a biological sample obtained from a "normal" or "healthy" individual that does not have cancer. A normal control sample can also be a sample that contains the same concentration of thymosin β 16 normally present in a biological sample obtained from a healthy individual that does not have cancer.

[0013] The term "test sample" refers to a biological sample obtained from a patient suspected of having cancer.

[0014] Biological samples include, for example, blood, tissue, serum, stool, urine, sputum, cerebrospinal fluid, nipple aspirate, and supernatant from cell lysate.

[0015] In another aspect, the present invention provides a method for prognostic evaluation of a patient suspected of having, or having, a cancer. The method comprises the steps of i) measuring the level of thymosin β 16 present in a test sample obtained from the patient, ii) comparing the level of thymosin β 16 in the test sample to a range of thymosin β 16 known to be present in biological samples of the same type, which are obtained from healthy patients that do not have cancer, and iii) evaluating the prognosis of the patient based on the comparison, where a high level of thymosin β 16 in the test sample indicates an aggressive form of cancer (e.g. metastatic or invasive) and therefore a poor prognosis.

[0016] Thymosin β 16 mRNA or protein may be measured to obtain thymosin β 16 levels.

[0017] The present invention also contemplates the assessment of the level of thymosin β 16 present in multiple test samples obtained from the same patient, where a progressive increase in the amount of thymosin β 16 over time indicates an increased aggressiveness (e.g. metastatic potential) of the cancer/tumor.

[0018] In the methods of the present invention, levels of thymosin β 16 can be ascertained by measuring the protein directly or indirectly by measuring mRNA transcript encoding thymosin β 16. mRNA levels can be measured, for example, using Northern blot analysis or an RNA dependent polymerase chain reaction, e.g., reverse transcriptase PCR (RT-PCR). DNA chip technology may also be used to measure mRNA levels.

[0019] The present invention also provides a method for measuring thymosin β 16 levels which comprises the steps of:

[0020] contacting a biological specimen with an antibody or antibody fragment which selectively binds thymosin β 16, and

[0021] detecting whether said antibody or said antibody fragment is bound by said sample and thereby measuring the levels of thymosin β 16.

[0022] In still another embodiment of this invention, the protein can serve as a target for agents that disrupt its function. Such agents include compounds or antibodies that bind to thymosin β 16 such that its function is inhibited. For example, one can add an effective amount of a compound that binds to thymosin β 16 to disrupt

function and thus inhibit metastasis. In another embodiment, one can use thymosin β 16 expressing cells in an assay to discover compounds that bind to or otherwise interact with this protein in order to discover compounds that can be used to inhibit metastasis.

[0023] In a further embodiment of the invention, thymosin β 16 or an immunogenic polypeptide thereof (or DNA encoding the protein or polypeptide) may be used in a pharmaceutical composition or vaccine to treat cancer or to inhibit the development of cancer.

[0024] In accordance with yet another aspect of the present invention, there are provided isolated antibodies or antibody fragments which selectively bind human thymosin β 16. The antibody fragments include, for example, Fab, Fab', F(ab')₂ or Fv fragments. The antibody may be a single chain antibody, a humanized antibody or a chimeric antibody.

[0025] The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, naturally-occurring polynucleotides or polypeptides present in a living animal are not isolated, but the same polynucleotides or DNA or polypeptides, separated from some or all of the coexisting materials in the natural system, are isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[0026] The present invention further provides a method of treating a neoplastic cell expressing human thymosin β 16 by administering to the cell an effective amount of a compound which suppresses the activity or production of the human thymosin β 16. Preferably, the compound interferes with the expression of the human thymosin β 16 gene. Such compounds include, for example, antisense oligonucleotides, si RNAs, ribozymes, RNAi, antibodies, including single chain antibodies and fragments thereof and aptamers.

[0027] As used herein, "thymosin β 16" means a protein having the amino acid sequence of SEQ ID NO:1.

[0028] As used herein, the term "unique fragment" refers to a portion of the thymosin β 16 nucleotide sequence or thymosin β 16 protein that contains sequences (either nucleotides or amino acid residues) present in thymosin β 16 (SEQ ID NOs: 1,

2, or 3) but not in other members of the thymosin family. This can be determined when the hybridization profile of that fragment under stringent conditions is such that it does not hybridize to other members of the thymosin family. Such fragments can also be ascertained by sequence comparison. Preferably, the unique nucleotide sequence fragment is at least 10 nucleotides in length, more preferably at least 20 nucleotides in length, most preferably at least 30 nucleotides in length. Preferably, the unique polypeptide sequence fragment is 4 to 20 amino acids in length, more preferably, 6 to 15 amino acids, most preferably, 6 to 10 amino acids.

[0029] Other aspects of the invention are disclosed infra.

BRIEF DESCRIPTION OF THE DRAWINGS

[0030] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate embodiments of the invention and, together with the description, serve to explain the objects, advantages, and principles of the invention.

[0031] Figure 1 shows a western blot showing the level of endogenous β -thymosin proteins (T β 4, T β 15, T β 16) in the LnCaP human prostate cancer cell line, originally derived from a lymph node metastasis, and in low or high metastatic variants of the Dunning rat prostatic carcinoma cell line.

[0032] Figure 2 shows a summary of the immunohistochemical staining data for T β 16 in human prostate specimens. Results for 10 specimens of benign prostatic hyperplasia and 51 prostatic carcinomas with Gleason scores ranging from 4 to 10 are shown. Each symbol represents the specimen from a different individual. Positive: samples with homogeneous staining of more than 75% of tumor cells. Parital: samples with heterogeneous staining of 10 – 75% of tumor cells. Negative: less than 10% of cells have a positive signal.

[0033] Figure 3 shows Northern blot analysis of β -thymosin RNA expression in low and high metastatic cell lines: carcinoma cell lines from pancreas; BxPC3 (Bx), prostate; Dunning Rat (Dunning), LnCaP, DU145 (DU), PC3M, and breast; MDA-MB 231 and 435 variants. Total RNA from parental (P) cell lines, or subclones that exhibit low (L) or high (H) metastatic properties in the SCID mouse model, were analyzed with β -thymosin specific or β -actin 32P labeled DNA probes.

[0034] Figure 4A-C shows T β 16 mRNA expression in normal human adult and fetal tissues. Figure 4A: Northern dot blot analysis of a Clontech RNA Master Blot (#7770-1) containing mRNA from human adult and fetal tissues using a T β 16 specific ³²P labeled DNA probe. Figure 4B: shows that type and position of polyA+ RNAs and controls dotted on nylon membrane in the Clontech RNA Master Blot (#7770-1). The range of T β 16 signal intensity for each tissue on the northern blot in Figure 4A is also indicated (+ to +++++). Figure 4C: shows Northern dot blot analysis of a Clontech RNA Master Blot (#7770-1) containing mRNA from human adult and fetal tissues using a β -actin specific ³²P labeled DNA probe.

[0035] Figure 5 shows a table of a variety of cancers found to have increased expression of T β 16. Increased expression of T β 16 as compared to non-cancerous tissue was monitored by (a) Antibody staining (b) Express Sequence Tag analysis, (c) Serial Analysis of Gene Expression (SAGE) (d) Gene Chip Array (e) or Northern Blot analysis.

[0036] Figure 6 shows Northern Blot analysis of T β 16 detected in various normal human tissues.

DETAILED DESCRIPTION OF THE INVENTION

[0037] We have discovered that thymosin β 16 is associated with prostate cancer but not BPH. Thus, expression of thymosin β 16 is useful as a diagnostic or prognostic indicator of prostate cancer, and other cancers, particularly cancers of epithelial origin. In addition, the protein provides a target for cancer treatment.

[0038] As used herein, "cancers of epithelial origin" refers to cancers that arise from epithelial cells which include, but are not limited to, breast cancer, basal cell carcinoma, adenocarcinoma, gastrointestinal cancer, lip cancer, mouth cancer, esophageal cancer, small bowel cancer and stomach cancer, colon cancer, liver cancer, bladder cancer, pancreas cancer, ovary cancer, cervical cancer, lung cancer, breast cancer and skin cancer, such as squamous cell and basal cell cancers, prostate cancer, renal cell carcinoma, and other known cancers that effect epithelial cells throughout the body.

[0039] The term "aggressive" or "invasive" with respect to cancer refers to the proclivity of a tumor for expanding beyond its boundaries into adjacent tissue (Darnell, J. (1990), Molecular Cell Biology, Third Ed., W. H. Freeman, NY). Invasive

cancer can be contrasted with organ-confined cancer wherein the tumor is confined to a particular organ. The invasive property of a tumor is often accompanied by the elaboration of proteolytic enzymes, such as collagenases, that degrade matrix material and basement membrane material to enable the tumor to expand beyond the confines of the capsule, and beyond confines of the particular tissue in which that tumor is located.

[0040] The term "metastasis", as used herein, refers to the condition of spread of cancer from the organ of origin to additional distal sites in the patient. The process of tumor metastasis is a multistage event involving local invasion and destruction of intercellular matrix, intravasation into blood vessels, lymphatics or other channels of transport, survival in the circulation, extravasation out of the vessels in the secondary site and growth in the new location (Fidler, et al., *Adv. Cancer Res.* 28, 149-250 (1978), Liotta, et al., *Cancer Treatment Res.* 40, 223-238 (1988), Nicolson, *Biochim. Biophys. Acta* 948, 175-224 (1988) and Zetter, *N. Eng. J. Med.* 322, 605-612 (1990)). Increased malignant cell motility has been associated with enhanced metastatic potential in animal as well as human tumors (Hosaka, et al., *Gann* 69, 273-276 (1978) and Haemmerlin, et al., *Int. J. Cancer* 27, 603-610 (1981)).

[0041] As used herein, a "biological sample" refers to a sample of biological material obtained from a patient, preferably a human patient, including a tissue, a tissue sample, a cell sample (e. g., a tissue biopsy, such as, an aspiration biopsy, a brush biopsy, a surface biopsy, a needle biopsy, a punch biopsy, an excision biopsy, an open biopsy, an incision biopsy or an endoscopic biopsy), and a tumor sample. Biological samples can also be biological fluid samples e.g., blood, urine, nipple aspirates.

[0042] The present invention also encompasses the use of isolates of a biological sample in the methods of the invention. As used herein, an "isolate" of a biological sample (e. g., an isolate of a tissue or tumor sample) refers to a material or composition (e. g., a biological material or composition) which has been separated, derived, extracted, purified or isolated from the sample and preferably is substantially free of undesirable compositions and/or impurities or contaminants associated with the biological sample.

[0043] As used herein, a "tissue sample" refers to a portion, piece, part, segment, or fraction of a tissue which is obtained or removed from an intact tissue of a subject, preferably a human subject. A preferred tissue sample is mammary tissue.

[0044] As used herein, a "tumor sample" refers to a portion, piece, part, segment, or fraction of a tumor, for example, a tumor which is obtained or removed from a subject (e. g., removed or extracted from a tissue of a subject), preferably a human subject.

[0045] As used herein, a "primary tumor" is a tumor appearing at a first site within the subject and can be distinguished from a "metastatic tumor" which appears in the body of the subject at a remote site from the primary tumor.

[0046] The present invention is directed to methods for diagnosis of cancer in a patient. The methods involve measuring levels of thymosin β 16 in a test sample obtained from a patient, suspected of having cancer, and comparing the observed levels to levels of thymosin β 16 found in a normal control sample, for example a sample obtained from a patient that does not have cancer. Levels of thymosin β 16 higher than levels that are observed in the normal control indicate the presence of cancer. The levels of thymosin β 16 can be represented by arbitrary units, for example as units obtained from a densitometer, luminometer, or an ELISA plate reader.

[0047] As used herein, "a higher level of thymosin β 16 in the test sample as compared to the level in the normal control sample" refers to an amount of thymosin β 16 that is greater than an amount of thymosin β 16 present in a normal control sample. The term "higher level" refers to a level that is statistically significant or significantly above levels found in the normal control sample. Preferably, the "higher level" is at least 2 fold greater.

[0048] The term "statistically significant" or "significantly" refers to statistical significance and generally means a two standard deviation (2SD) above normal, or higher, concentration of the marker.

[0049] As used herein, "a high level" of thymosin β 16 refers to amounts of thymosin β 16 that are at least 3 fold greater than the amounts of thymosin β 16 present in normal control samples, preferably 5 fold to 6 fold greater.

[0050] For purposes of comparison, the test sample and normal control sample are of the same type, that is, obtained from the same biological source. The normal control sample can also be a standard sample that contains the same concentration of thymosin β 16 that is normally found in a biological sample of the same type and that is obtained from a healthy individual. For example, there can be a standard normal control sample for the amounts of thymosin β 16 normally found in biological samples such as urine, blood, cerebral spinal fluid, or tissue.

[0051] Additionally, disease progression can be assessed by following thymosin β 16 levels in individual patients over time. Cancers include, for example, prostate cancer, lung carcinoma, breast carcinoma, thyroid carcinoma, brain cancers (cerebellum, medulloblastoma, astrocytoma, ependymoma, glioblastoma), pancreatic carcinoma, ovarian carcinoma, eye cancer (retinoblastoma), muscle (rhabdosarcoma), lymphoma, stomach cancer, liver cancer, colon cancer, kidney cancer.

[0052] The present invention further provides for methods of prognostic evaluation of a patient suspected of having, or having, cancer. The method comprises measuring the level of thymosin β 16 present in a test biological sample obtained from a patient and comparing the observed level with a range of thymosin β 16 levels normally found in biological samples (of the same type) of healthy individuals. A high level for example, is indicative of a greater potential for metastatic activity and corresponds to a poor prognosis, while lower levels indicate that the tumor is less aggressive and correspond to a better prognosis.

[0053] This information can be used by the physician in determining the most effective course of treatment. A course of treatment refers to the therapeutic measures taken for a patient after diagnosis or after treatment for cancer. For example, a determination of the likelihood for cancer recurrence, spread, or patient survival, can assist in determining whether a more conservative or more radical approach to therapy should be taken, or whether treatment modalities should be combined. For example, when cancer recurrence is likely, it can be advantageous to precede or follow surgical treatment with chemotherapy, radiation, immunotherapy, biological modifier therapy, gene therapy, vaccines, and the like, or adjust the span of time during which the patient is treated.

[0054] Changes in a patient's condition can be monitored using the methods of the present invention by comparing changes in thymosin β 16 expression levels in the tumor in that subject over time.

[0055] Biological specimens include, for example, blood, tissue, serum, stool, urine, sputum, nipple aspirates, cerebrospinal fluid and supernatant from cell lysate. Preferably, one uses tissue specimens, serum or urine. The determination of, and comparison of, thymosin β 16 levels is by standard modes of analysis based upon the present disclosure.

[0056] The methods of the invention can also be practiced, for example, by selecting a combination of thymosin β 16 and one or more biomarkers for which increased or decreased expression correlates with cancer, such as any of thymosin β 15 (See for example PCT publication WO 97/48805), thymosin β 4, thymosin β 10, cIAP2, Apaf1, Bcl-2, Smac, or another known or standard biomarker for cancer. The selected biomarker can be a general diagnostic or prognostic marker useful for multiple types of cancer, such as CA 125, CEA or LDH, or can be a cancer-specific diagnostic or prognostic marker, such as a colon cancer marker (for example, sialosyl-TnCEA, CA19-9, or LASA), breast cancer marker (for example, CA 15-2. Her-2/neu and CA 27.29), ovarian cancer marker (for example, CA72-4), lung cancer (for example, neuron-specific enolase (NSE) and tissue polypeptide antigen (TPA)), prostate cancer (for example, PSA, prostate-specific membrane antigen and prostatic acid phosphatase), melanoma (for example, S-100 and TA-90), as well as other biomarkers specific for other types of cancer. Those skilled in the art will be able to select useful diagnostic or prognostic markers for detection in combination with thymosin β 16. Similarly, three or more, four or more or five or more or a multitude of biomarkers can be used together for determining a diagnosis or prognosis of a patient.

Thymosin β 16 Detection Techniques

[0057] The present invention features agents which are capable of detecting Thymosin β 16 polypeptide or mRNA such that the presence of Thymosin β 16 is detected and/or quantitated. As defined herein, an "agent" refers to a substance which is capable of identifying or detecting Thymosin β 16 in a biological sample (e. g., identifies or detects Thymosin β 16 mRNA, Thymosin β 16 DNA, Thymosin β 16 protein). In one embodiment, the agent is a labeled or labelable antibody which specifically binds to Thymosin β 16 polypeptide. As used herein, the phrase "labeled or labelable" refers to the attaching or including of a label (e. g., a marker or indicator) or ability to attach or include include a label (e. g., a marker or indicator). Markers or indicators include, but are not limited to, for example, radioactive molecules, colorimetric molecules, and enzymatic molecules which produce detectable changes in a substrate.

[0058] In one embodiment the agent is an antibody which specifically binds to all or a portion of a Thymosin β 16 protein. As used herein, the phrase "specifically

binds" refers to binding of, for example, an antibody to an epitope or antigen or antigenic determinant in such a manner that binding can be displaced or competed with a second preparation of identical or similar epitope, antigen or antigenic determinant. In an exemplary embodiment, the agent is an antibody which specifically binds to all or a portion of the human Thymosin β 16 protein. The term "specifically binds" means that the antibody binds only to Thymosin β 16 protein and not other members of the thymosin family, such as Thymosin β 10, Thymosin β 4, or Thymosin β 15.

[0059] In yet another embodiment the agent is a labeled or labelable nucleic acid probe capable of hybridizing to Thymosin β 16 mRNA. For example, the agent can be an oligonucleotide primer for the polymerase chain reaction which flank or lie within the nucleotide sequence encoding human Thymosin β 16. In a preferred embodiment, the biological sample being tested is an isolate, for example, RNA. In yet another embodiment, the isolate (e. g., the RNA) is subjected to an amplification process which results in amplification of Thymosin β 16 nucleic acid. As defined herein, an "amplification process" is designed to strengthen, increase, or augment a molecule within the isolate. For example, where the isolate is mRNA, an amplification process such as RT-PCR can be utilized to amplify the mRNA, such that a signal is detectable or detection is enhanced. Such an amplification process is beneficial particularly when the biological, tissue, or tumor sample is of a small size or volume.

[0060] Standard detection techniques well known in the art for detecting RNA, DNA, proteins and peptides can readily be applied to detect thymosin β 16 or its transcript to diagnose cancer, especially metastatic cancer, or to confirm that a primary tumor has, or has not, reached a particular metastatic phase. Such techniques may include detection with nucleotide probes or may comprise detection of the protein by, for example, antibodies or their equivalent. Preferably, the nucleotide probes hybridize to the sequence shown in SEQ ID Nos:2 or 3 for thymosin β 16.

Thymosin β 16 Nucleic Acid Probes

[0061] Types of probe include cDNA, riboprobes, synthetic oligonucleotides and genomic probes. The type of probe used will generally be dictated by the particular situation, such as riboprobes for in situ hybridization, and cDNA for Northern blotting, for example. Most preferably, the probe is directed to nucleotide regions

unique to the protein. Detection of the thymosin β 16 encoding gene, per se, will be useful in screening for mutations associated with enhanced expression. Other forms of assays to detect targets more readily associated with levels of expression -- transcripts and other expression products-- will generally be useful as well. The probes may be as short as is required to differentially recognize thymosin β 16 mRNA transcripts, and may be as short as, for example, 15 bases; however, probes of at least 17 bases, more preferably 18 bases and still more preferably 20 bases are preferred.

[0062] A probe may also be reverse-engineered by one skilled in the art from the amino acid sequence of SEQ ID NO: 1. However use of such probes may be more limited than the native DNA sequence, as it will be appreciated that any one given reverse-engineered sequence will not necessarily hybridize well, or at all, with any given complementary sequence reverse-engineered from the same peptide, owing to the degeneracy of the genetic code. This is a factor common in the calculations of those skilled in the art, and the degeneracy of any given sequence is frequently so broad as to yield a large number of probes for any one sequence.

[0063] The form of labeling of the probes may be any that is appropriate, such as the use of radioisotopes, for example, ^{32}P and ^{35}S . Labeling with radioisotopes may be achieved, whether the probe is synthesized chemically or biologically, by the use of suitably labeled bases.

Thymosin β 16 RNA Detection Techniques

[0064] Detection of RNA transcripts may be achieved by Northern blotting, for example, wherein a preparation of RNA is run on a denaturing agarose gel, and transferred to a suitable support, such as activated cellulose, nitrocellulose or glass or nylon membranes. Radiolabeled cDNA or RNA is then hybridized to the preparation, washed and analyzed by autoradiography.

[0065] Detection of RNA transcripts can further be accomplished using known amplification methods. For example, it is within the scope of the present invention to reverse transcribe mRNA into cDNA followed by polymerase chain reaction (RT-PCR); or, to use a single enzyme for both steps as described in U.S. Pat. No. 5,322,770, or reverse transcribe mRNA into cDNA followed by symmetric gap ligase chain reaction (RT-AGLCR) as described by R. L. Marshall, et al., PCR Methods and Applications 4: 80-84 (1994).

[0066] Other known amplification methods which can be utilized herein include but are not limited to the so-called "NASBA" or "3SR" technique described in PNAS USA 87: 1874-1878 (1990) and also described in Nature 350 (No. 6313): 91-92 (1991); Q-beta amplification as described in published European Patent Application (EPA) No. 4544610; strand displacement amplification (as described in G. T. Walker et al., Clin. Chem. 42: 9-13 (1996) and European Patent Application No. 684315; and target mediated amplification, as described by PCT Publication WO 9322461.

[0067] In situ hybridization visualization may also be employed, wherein a radioactively labeled antisense RNA probe is hybridized with a thin section of a biopsy sample, washed, cleaved with RNase and exposed to a sensitive emulsion for autoradiography. The samples may be stained with haematoxylin to demonstrate the histological composition of the sample, and dark field imaging with a suitable light filter shows the developed emulsion. Non-radioactive labels such as digoxigenin may also be used.

[0068] Alternatively, mRNA expression can be detected on a DNA array, chip or a microarray. Oligonucleotides corresponding to the thymosin β 16 are immobilized on a chip which is then hybridized with labeled nucleic acids of a test sample obtained from a patient. Positive hybridization signal is obtained with the sample containing thymosin β 16 transcripts. Methods of preparing DNA arrays and their use are well known in the art. (See, for example U.S. Patent NOs: 6,618,6796; 6,379,897; 6,664,377; 6,451,536; 548,257; U.S. 20030157485 and Schena et al. 1995 Science 20:467-470; Gerhold et al. 1999 Trends in Biochem. Sci. 24, 168-173; and Lennon et al. 2000 Drug discovery Today 5: 59-65, which are herein incorporated by reference in their entirety). Serial Analysis of Gene Expression (SAGE) can also be performed (See for example U.S. Patent Application 20030215858).

[0069] To monitor mRNA levels, for example, mRNA is extracted from the biological sample to be tested, reverse transcribed, and fluorescent-labeled cDNA probes are generated. The microarrays capable of hybridizing to thymosin β 16 cDNA are then probed with the labeled cDNA probes, the slides scanned and fluorescence intensity measured. This intensity correlates with the hybridization intensity and expression levels.

Thymosin β 16 Antibodies

[0070] Antibodies may be raised against either a peptide of thymosin β 16 or the whole molecule. For example, a peptide may be presented together with a carrier protein, such as an KLH, to an animal system or, if it is long enough, say 25 amino acid residues, without a carrier. Antibodies can also be raised against homologs or orthologs of Thymosin β 16. Modified Thymosin β 16 proteins may also be used, for example chemically modified proteins (e.g. methylation, acetylation, or others), fusion proteins, or mutants. All that is required is that the antibody produced specifically binds to Thymosin β 16.

[0071] Polyclonal antibodies generated by the above technique may be used directly, or suitable antibody producing cells may be isolated from the animal and used to form a hybridoma by known means (Kohler and Milstein, Nature 256:795. (1975)). Selection of an appropriate hybridoma will also be apparent to those skilled in the art, and the resulting antibody may be used in a suitable assay to identify thymosin β 16.

[0072] The term "antibody" as used herein encompasses polyclonal or monoclonal antibodies as well as functional fragments of antibodies, including fragments of chimeric, human, humanized, primatized, veneered or single-chain antibodies. Functional fragments include antigen-binding fragments which bind to thymosin β 16. For example, antibody fragments capable of binding to thymosin β 16 or portions thereof, including, but not limited to Fv, Fab, Fab' and F (ab')₂ fragments can be used. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or F (ab')₂ fragments, respectively. Other proteases with the requisite substrate specificity can also be used to generate Fab or F (ab')₂ fragments. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. For example, a chimeric gene encoding a F (ab')₂ heavy chain portion can be designed to include DNA sequences encoding the CH₁ domain and hinge region of the heavy chain.

[0073] Single-chain antibodies, and chimeric, human, humanized or primatized (CDR-grafted), or veneered antibodies, as well as chimeric, CDR-grafted or veneered single-chain antibodies, comprising portions derived from different species, and the like are also encompassed by the present invention and the term "antibody". The various portions of these antibodies can be joined together chemically by

conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques. For example, nucleic acids encoding a chimeric or humanized chain can be expressed to produce a contiguous protein. See, e.g., Cabilly et al., U. S. Patent No. 4, 816, 567 ; Cabilly et al., European Patent No. 0,125,023 B1; Boss et al., U. S. Patent No. 4,816,397; Boss et al., European Patent No. 0,120,694 B1; Neuberger, M. S. et al., WO 86/01533; Neuberger, M. S. et al., European Patent No. 0,194,276 B1; Winter, U. S. Patent No. 5,225,539; Winter, European Patent No. 0,239,400 B1; Queen et al., European Patent No. 0451216 B1 ; and Padlan, E. A. et al., EP 0519596 A1. See also, Newman, R. et al., *BioTechnology*, 10: 1455-1460 (1992), regarding primatized antibody, and Ladner et al., U. S. Patent No. 4,946,778 and Bird, R. E. et al., *Science*, 242: 423-426 (1988)) regarding single-chain antibodies.

[0074] Preparation of immunizing antigen, and polyclonal and monoclonal antibody production can be performed using any suitable technique. For example, monoclonal antibodies directed against binding cell surface epitopes can be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Other suitable methods of producing or isolating antibodies of the requisite specificity can be used, including, for example, methods which select recombinant antibody from a library (e.g., a phage display library).

[0075] In some embodiments, agents that specifically bind to Thymosin β 16 other than antibodies are used, such as peptides. Peptides that specifically bind to Thymosin β 16 can be identified by any means known in the art. For example, specific peptide binders of Thymosin β 16 can be screened for using peptide phage display libraries.

Thymosin β 16 Protein Detection Techniques

[0076] It is generally preferred to use antibodies, or antibody equivalents, to detect thymosin β 16 protein. Methods for the detection of protein are well known to those skilled in the art, and include ELISA (enzyme linked immunosorbent assay), RIA (radioimmunoassay), Western blotting, and immunohistochemistry. Immunoassays such as ELISA or RIA, which can be extremely rapid, are more generally preferred. Antibody arrays or protein chips can also be employed, see for

example U.S. Patent Application Nos: 20030013208A1; 20020155493A1, 20030017515 and U.S. Patent Nos: 6,329,209; 6,365,418, herein incorporated by reference in their entirety.

[0077] Samples for diagnostic purposes may be obtained from any number of sources. A sample obtained directly from the tumor, such as the stroma or cytosol, may be used to determine the metastatic potential of the tumor. It may also be appropriate to obtain the sample from other biological specimens, such as blood, lymph nodes, or urine. Such diagnosis may be of particular importance in monitoring progress of a patient, such as after surgery to remove a tumor. If a reference reading is taken after the operation, then another taken at regular intervals, any rise could be indicative of a relapse, or possibly a metastasis.

[0078] ELISA and RIA procedures may be conducted such that a thymosin β 16 standard is labeled (with a radioisotope such as ^{125}I or ^{35}S , or an assayable enzyme, such as horseradish peroxidase or alkaline phosphatase), and, together with the unlabelled sample, brought into contact with the corresponding antibody, whereon a second antibody is used to bind the first, and radioactivity or the immobilized enzyme assayed (competitive assay). Alternatively, thymosin β 16 in the sample is allowed to react with the corresponding immobilized antibody, radioisotope- or enzyme-labeled anti-thymosin β 16 antibody is allowed to react with the system, and radioactivity or the enzyme assayed (ELISA-sandwich assay). Other conventional methods may also be employed as suitable.

[0079] The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting antigen with immobilized antibody and, without washing, contacting the mixture with labeled antibody. The "two-step" assay involves washing before contacting the mixture with labeled antibody. Other conventional methods may also be employed as suitable.

[0080] Enzymatic and radiolabeling of thymosin β 16 and/or the antibodies may be effected by conventional means. Such means will generally include covalent linking of the enzyme to the antigen or the antibody in question, such as by glutaraldehyde, specifically so as not to adversely affect the activity of the enzyme, by which is meant that the enzyme must still be capable of interacting with its substrate, although it is not necessary for all of the enzyme to be active, provided that enough remains active to permit the assay to be effected. Indeed, some techniques for binding

enzyme are non-specific (such as using formaldehyde), and will only yield a proportion of active enzyme.

[0081] It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the system to be brought into contact with the component and readily removed without laborious and time-consuming labor. It is possible for a second phase to be immobilized away from the first, but one phase is usually sufficient.

[0082] It is possible to immobilize the enzyme itself on a support, but if solid-phase enzyme is required, then this is generally best achieved by binding to antibody and affixing the antibody to a support, models and systems for which are well-known in the art. Simple polyethylene may provide a suitable support.

[0083] Enzymes employable for labeling are not particularly limited, but may be selected from the members of the oxidase group, for example. These catalyze production of hydrogen peroxide by reaction with their substrates, and glucose oxidase is often used for its good stability, ease of availability and cheapness, as well as the ready availability of its substrate (glucose). Activity of the oxidase may be assayed by measuring the concentration of hydrogen peroxide formed after reaction of the enzyme-labeled antibody with the substrate under controlled conditions well-known in the art.

[0084] Other techniques may be used to detect thymosin β 16 according to a practitioner's preference based upon the present disclosure. One such technique is Western blotting (Towbin et al., Proc. Nat. Acad. Sci. 76:4350 (1979)), wherein a suitably treated sample is run on an SDS-PAGE gel before being transferred to a solid support, such as a nitrocellulose filter. Anti-thymosin β 16 antibodies (unlabeled) are then brought into contact with the support and assayed by a secondary immunological reagent, such as labeled protein A or anti-immunoglobulin (suitable labels including ^{125}I , horseradish peroxidase and alkaline phosphatase). Chromatographic detection may also be used.

[0085] Immunohistochemistry may be used to detect expression of human thymosin β 16 in a biopsy sample. A suitable antibody is brought into contact with, for example, a thin layer of cells, washed, and then contacted with a second, labeled antibody. Labeling may be by fluorescent markers, enzymes, such as peroxidase, avidin, or radiolabeling. The assay is scored visually, using microscopy.

[0086] In addition, the Thymosin β 16 protein may be detected using Mass Spectrometry such as MALDI/TOF (time-of-flight), SELDI/TOF, liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), high performance liquid chromatography-mass spectrometry (HPLC-MS), capillary electrophoresis-mass spectrometry, nuclear magnetic resonance spectrometry, or tandem mass spectrometry (e.g., MS/MS, MS/MS/MS, ESI-MS/MS, etc.). See for example, U.S. Patent Application Nos: 20030199001, 20030134304, 20030077616, which are herein incorporated by reference.

[0087] Mass spectrometry methods are well known in the art and have been used to quantify and/or identify biomolecules, such as proteins (see, e.g., Li et al. (2000) *Tibtech* 18:151-160; Rowley et al. (2000) *Methods* 20: 383-397; and Kuster and Mann (1998) *Curr. Opin. Structural Biol.* 8: 393-400). Further, mass spectrometric techniques have been developed that permit at least partial de novo sequencing of isolated proteins. Chait et al., *Science* 262:89-92 (1993); Keough et al., *Proc. Natl. Acad. Sci. USA.* 96:7131-6 (1999); reviewed in Bergman, *EXS* 88:133-44 (2000).

[0088] In certain embodiments, a gas phase ion spectrophotometer is used. In other embodiments, laser-desorption/ionization mass spectrometry is used to analyze the sample. Modern laser desorption/ionization mass spectrometry ("LDI-MS") can be practiced in two main variations: matrix assisted laser desorption/ionization ("MALDI") mass spectrometry and surface-enhanced laser desorption/ionization ("SELDI"). In MALDI, the analyte is mixed with a solution containing a matrix, and a drop of the liquid is placed on the surface of a substrate. The matrix solution then co-crystallizes with the biological molecules. The substrate is inserted into the mass spectrometer. Laser energy is directed to the substrate surface where it desorbs and ionizes the biological molecules without significantly fragmenting them. However, MALDI has limitations as an analytical tool. It does not provide means for fractionating the sample, and the matrix material can interfere with detection, especially for low molecular weight analytes. See, e.g., U.S. Pat. No. 5,118,937 (Hillenkamp et al.), and U.S. Pat. No. 5,045,694 (Beavis & Chait).

[0089] In SELDI, the substrate surface is modified so that it is an active participant in the desorption process. In one variant, the surface is derivatized with adsorbent and/or capture reagents that selectively bind the protein of interest. In another variant, the surface is derivatized with energy absorbing molecules that are

not desorbed when struck with the laser. In another variant, the surface is derivatized with molecules that bind the protein of interest and that contain a photolytic bond that is broken upon application of the laser. In each of these methods, the derivatizing agent generally is localized to a specific location on the substrate surface where the sample is applied. See, e.g., U.S. Pat. No. 5,719,060 (Hutchens & Yip) and WO 98/59361 (Hutchens & Yip). The two methods can be combined by, for example, using a SELDI affinity surface to capture an analyte and adding matrix-containing liquid to the captured analyte to provide the energy absorbing material.

[0090] For additional information regarding mass spectrometers, see, e.g., Principles of Instrumental Analysis, 3rd edition., Skoog, Saunders College Publishing, Philadelphia, 1985; and Kirk-Othmer Encyclopedia of Chemical Technology, 4.sup.th ed. Vol. 15 (John Wiley & Sons, New York 1995), pp. 1071-1094.

[0091] Detection of the presence of a marker or other substances will typically involve detection of signal intensity. This, in turn, can reflect the quantity and character of a polypeptide bound to the substrate. For example, in certain embodiments, the signal strength of peak values from spectra of a first sample and a second sample can be compared (e.g., visually, by computer analysis etc.), to determine the relative amounts of particular biomolecules. Software programs such as the Biomarker Wizard program (CIPHERGEN Biosystems, Inc., Fremont, Calif.) can be used to aid in analyzing mass spectra. The mass spectrometers and their techniques are well known to those of skill in the art.

[0092] Any person skilled in the art understands, any of the components of a mass spectrometer (e.g., desorption source, mass analyzer, detect, etc.) and varied sample preparations can be combined with other suitable components or preparations described herein, or to those known in the art. For example, in some embodiments a control sample may contain heavy atoms (e.g. ^{13}C) thereby permitting the test sample to mixed with the known control sample in the same mass spectrometry run.

[0093] In one preferred embodiment, a laser desorption time-of-flight (TOF) mass spectrometer is used. In laser desorption mass spectrometry, a substrate with a bound marker is introduced into an inlet system. The marker is desorbed and ionized into the gas phase by laser from the ionization source. The ions generated are collected by an ion optic assembly, and then in a time-of-flight mass analyzer, ions are accelerated through a short high voltage field and let drift into a high vacuum chamber. At the far end of the high vacuum chamber, the accelerated ions strike a

sensitive detector surface at a different time. Since the time-of-flight is a function of the mass of the ions, the elapsed time between ion formation and ion detector impact can be used to identify the presence or absence of molecules of specific mass to charge ratio.

[0094] In some embodiments the relative amounts of one or more biomolecules present in a first or second sample is determined, in part, by executing an algorithm with a programmable digital computer. The algorithm identifies at least one peak value in the first mass spectrum and the second mass spectrum. The algorithm then compares the signal strength of the peak value of the first mass spectrum to the signal strength of the peak value of the second mass spectrum of the mass spectrum. The relative signal strengths are an indication of the amount of the biomolecule that is present in the first and second samples. A standard containing a known amount of a biomolecule can be analyzed as the second sample to provide better quantify the amount of the biomolecule present in the first sample. In certain embodiments, the identity of the biomolecules in the first and second sample can also be determined.

Thymosin β 16 Detection Kit

[0095] This invention provides a convenient kit for measuring human thymosin β 16. This kit includes antibodies or antibody fragments which selectively bind human thymosin β 16 or a set of DNA oligonucleotide primers that allows synthesis of cDNA encoding the protein or a DNA probe that detects expression of thymosin β 16 mRNA. Preferably, the primers and probes comprise at least 17 nucleotides and hybridize under stringent conditions to a DNA fragment having the nucleotide sequence set forth in SEQ ID NOS: 2 or 3. As herein used, the term "stringent conditions" means hybridization will occur only if there is at least 95% and preferably at least 97% identity between the sequences.

Thymosin β 16 DNA and Protein Production

[0096] DNA encoding thymosin β 16 and recombinant thymosin β 16 may be produced according to the methods known in the art.

[0097] The thymosin β 16 protein may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods. Proteins can be produced by well known synthetic methods

including Merrifield solid-phase synthesis, t-Boc-based synthesis, Fmoc synthesis and variations on these techniques (See, for example, Atherton and Sheppard, *Solid Phase Peptide Synthesis: A Practical Approach*, New York: IRL Press, 1989; Stewart and Young: *Solid- Phase Peptide Synthesis* 2nd Ed., Rockford, Ill.: Pierce Chemical Co., 1984; and Jones, *The Chemical Synthesis of Peptides*, Oxford: Clarendon Press, 1994). Recombinant methods are one preferred method to produce the thymosin β 16 protein. A wide variety of molecular and biochemical methods are available for generating and expressing the thymosin β 16; see e.g. the procedures disclosed in *Molecular Cloning, A Laboratory Manual* (2nd Ed., Sambrook, Fritsch and Maniatis, Cold Spring Harbor), *Current Protocols in Molecular Biology* (Eds. Ausubel, Brent, Kingston, More, Feidman, Smith and Stuhl, Greene Publ. Assoc., Wiley-Interscience, NY, N.Y. 1992) or other procedures that are otherwise known in the art.

Thymosin β 16 Cloning

[0098] Where it is desired to express the protein or a fragment thereof, any suitable system can be used. The general nature of suitable vectors, expression vectors and constructions therefor will be apparent to those skilled in the art.

[0099] Suitable expression vectors may be based on phages or plasmids, both of which are generally host-specific, although these can often be engineered for other hosts. Other suitable vectors include cosmids and retroviruses, and any other vehicles, which may or may not be specific for a given system. Control sequences, such as recognition, promoter, operator, inducer, terminator and other sequences essential and/or useful in the regulation of expression, will be readily apparent to those skilled in the art.

[00100] Correct preparation of nucleotide sequences may be confirmed, for example, by the method of Sanger et al. (*Proc. Natl. Acad. Sci. USA* 74:5463-7 (1977)).

[00101] A DNA fragment encoding the thymosin β 16 or a fragment thereof, may readily be inserted into a suitable vector. Ideally, the receiving vector has suitable restriction sites for ease of insertion, but blunt-end ligation, for example, may also be used, although this may lead to uncertainty over reading frame and direction of insertion. In such an instance, it is a matter of course to test transformants for expression, 1 in 6 of which should have the correct reading frame. Suitable vectors

may be selected as a matter of course by those skilled in the art according to the expression system desired.

Thymosin β 16 Protein Production

[00102] Isolated thymosin β 16 protein and fragments thereof may be produced using any expression system known to those skilled in the art. Such suitable expression systems include bacteria, such as *E. coli*, and eukaryotes, such as yeast, baculovirus, insect or mammalian cell-based expression systems, etc., depending on the size, nature and quantity of the polypeptide.

[00103] The term "isolated" means that the polypeptide is removed from its original environment. For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or DNA or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[00104] By transforming a suitable bacterial or eukaryotic organism, and preferably, a eukaryotic cell line, such as HeLa cells, with the plasmid obtained, selecting the eukaryotic transformant with geneticin, zeocin, blasticin or a similar compound (or ampicillin in the case of a bacterial transformant) or by other suitable means if required, and adding tryptophan or other suitable promoter-inducer if necessary, the desired polypeptide or protein may be expressed. The extent of expression may be analyzed by SDS polyacrylamide gel electrophoresis-SDS-PAGE (Laemelli, Nature 227:680-685 (1970)).

[00105] Suitable methods for growing and transforming cultures are usefully illustrated in, for example, Maniatis (Molecular Cloning, A Laboratory Notebook, Maniatis et al. (eds.), Cold Spring Harbor Labs, N.Y. (1989)).

[00106] Cultures useful for production of polypeptides or proteins may suitably be cultures of any living cells, and may vary from prokaryotic expression systems up to eukaryotic expression systems. One preferred prokaryotic system is that of *E. coli*, owing to its ease of manipulation. However, it is also possible to use a higher system, such as a mammalian cell line, for expression of a eukaryotic protein. Currently preferred cell lines for transient expression are the HeLa and Cos cell lines. Other

expression systems include the Chinese Hamster Ovary (CHO) cell line and the baculovirus system.

[00107] Other expression systems which may be employed include streptomycetes, for example, and yeasts, such as *Saccharomyces* spp., especially *S. cerevisiae*. Any system may be used as desired, generally depending on what is required by the operator. Suitable systems may also be used to amplify the genetic material, but it is generally convenient to use *E. coli* for this purpose when only proliferation of the DNA is required.

[00108] The polypeptides and proteins may be isolated from the fermentation or cell culture and purified using any of a variety of conventional methods including: liquid chromatography such as normal or reversed phase, using HPLC, FPLC and the like; affinity chromatography (such as with inorganic ligands or monoclonal antibodies); size exclusion chromatography; immobilized metal chelate chromatography; gel electrophoresis; and the like. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

Therapeutic Applications Using thymosin β 16

[00109] The presence of thymosin β 16 protein is positively correlated with metastasis. Therefore, thymosin β 16 protein could be useful in therapeutic and diagnostic applications. For example, therapeutic approaches include the use of antibodies to block thymosin β 16 protein, the use of antibodies for imaging applications, antisense, and RNAi technology to block thymosin β 16 expression, membrane localization for tumor targeting and delivery of therapeutics to tumor cells, and immunotherapies such as vaccines. Diagnostically, the cleavage of ectodomain of the thymosin β 16 protein, easily detected in the blood serum or urine, can be used as a marker for metastatic cancer.

Thymosin β 16 Blocking Antibodies or Aptamers

[00110] One can treat a range of afflictions or diseases associated with expression of the protein by directly blocking the protein. This can be accomplished by a range of different approaches, including the use of antibodies, small molecules, and

antagonists. One preferred approach is the use of antibodies that specifically block activity of the protein. Aptamers may also be used.

[00111] In accordance with yet another aspect of the present invention, there are provided isolated antibodies or antibody fragments which selectively bind the protein. The antibody fragments include, for example, Fab, Fab', F(ab')₂ or Fv fragments. The antibody may be a single chain antibody, a humanized antibody or a chimeric antibody.

[00112] Antibodies, or their equivalents, or other thymosin β 16 antagonists may also be used in accordance with the present invention for the treatment or prophylaxis of cancers. Administration of a suitable dose of the antibody or the antagonist may serve to block the activity of the protein and this may provide a crucial time window in which to treat the malignant growth.

[00113] Prophylaxis may be appropriate even at very early stages of the disease, as it is not known what specific event actually triggers metastasis in any given case. Thus, administration of the antibodies, their equivalents, intrabodies, antagonists which interfere with protein activity, may be effected as soon as cancer is diagnosed, and treatment continued for as long as is necessary, preferably until the threat of the disease has been removed. Such treatment may also be used prophylactically in individuals at high risk for development of certain cancers, e.g., prostate or breast.

[00114] A method of treatment involves attachment of a suitable toxin to the antibodies which then target the area of the tumor. Such toxins are well known in the art, and may comprise toxic radioisotopes, heavy metals, enzymes and complement activators, as well as such natural toxins as ricin which are capable of acting at the level of only one or two molecules per cell. It may also be possible to use such a technique to deliver localized doses of suitable physiologically active compounds, which may be used, for example, to treat cancers.

[00115] It will be appreciated that antibodies for use in accordance with the present invention, whether for diagnostic or therapeutic applications, may be monoclonal or polyclonal as appropriate. Antibody equivalents of these may comprise: the Fab' fragments of the antibodies, such as Fab, Fab', F(ab')₂ and Fv; idiotoxes; or the results of allotope grafting (where the recognition region of an animal antibody is grafted into the appropriate region of a human antibody to avoid an immune response in the patient), for example. Single chain antibodies may also be

used. Other suitable modifications and/or agents will be apparent to those skilled in the art.

[00116] Chimeric and humanized antibodies are also within the scope of the invention. It is expected that chimeric and humanized antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody. A variety of approaches for making chimeric antibodies, comprising for example a non-human variable region and a human constant region, have been described. See, for example, Morrison et al., Proc. Natl. Acad. Sci. U.S.A. 81,6851 (1985); Takeda, et al., Nature 314,452(1985), Cabilly et al., U.S. Pat. No. 4,816,567; Boss et al., U.S. Pat. No. 4,816,397; Tanaguchi et al., European Patent Publication EP 171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. Additionally, a chimeric antibody can be further "humanized" such that parts of the variable regions, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

[00117] In addition to using antibodies to inhibit thymosin β 16, it may also be possible to use other forms of inhibitors. For example, it may be possible to identify antagonists that functionally inhibit thymosin β 16. In addition, it may also be possible to interfere with the binding of thymosin β 16 to target proteins. Other suitable inhibitors will be apparent to the skilled person.

[00118] The present invention further provides use of the thymosin β 16 for intracellular or extracellular targets to affect activity. Intracellular targeting can be accomplished through the use of intracellularly expressed antibodies referred to as intrabodies.

[00119] The antibody (or other inhibitors or intrabody) can be administered by a number of methods. One preferred method is set forth by Marasco and Haseltine in PCT WO94/02610, which is incorporated herein by reference. This method discloses

the intracellular delivery of a gene encoding the antibody. One would preferably use a gene encoding a single chain antibody. The antibody would preferably contain a nuclear localization sequence. One preferably uses an SV40 nuclear localization signal. By this method one can intracellularly express an antibody, which can block thymosin β 16 functioning in desired cells.

[00120] Where the present invention provides for the administration of, for example, antibodies to a patient, then this may be by any suitable route. If the tumor is still thought to be, or diagnosed as, localized, then an appropriate method of administration may be by injection direct to the site. Administration may also be by injection, including subcutaneous, intramuscular, intravenous and intradermal injections.

[00121] Aptamer can be produced using the methodology disclosed in a U.S. Patent No. 5,270,163 and WO 91/19813.

[00122] Formulations may be any that are appropriate to the route of administration, and will be apparent to those skilled in the art. The formulations may contain a suitable carrier, such as saline, and may also comprise bulking agents, other medicinal preparations, adjuvants and any other suitable pharmaceutical ingredients. Catheters are one preferred mode of administration.

Imaging Techniques

[00123] Anti-thymosin β 16 antibodies may also be used for imaging purposes, for example, to detect tumor metastasis. Suitable labels include radioisotopes, iodine (^{125}I , ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^3H), indium (^{112}In), and technetium ($^{99\text{m}}\text{Tc}$), fluorescent labels, such as fluorescein and rhodamine, and biotin.

[00124] However, for in vivo imaging purposes, the position becomes more restrictive, as antibodies are not detectable, as such, from outside the body, and so must be labeled, or otherwise modified, to permit detection. Markers for this purpose may be any that do not substantially interfere with the antibody binding, but which allow external detection. Suitable markers may include those that may be detected by X-radiography, NMR or MRI. For X-radiographic techniques, suitable markers include any radioisotope that emits detectable radiation but that is not overtly harmful to the patient, such as barium or caesium, for example. Suitable markers for NMR and MRI generally include those with a detectable characteristic spin, such as deuterium,

which may be incorporated into the antibody by suitable labeling of nutrients for the relevant hybridoma, for example.

[00125] The size of the subject, and the imaging system used, will determine the quantity of imaging moiety needed to produce diagnostic images. In the case of a radioisotope moiety, for a human subject, the quantity of radioactivity injected will normally range from about 5 to 20 millicuries of technetium-99m. The labeled antibody or antibody fragment will then preferentially accumulate at the location of cells which contain thymosin β 16. The labeled antibody or antibody fragment can then be detected using known techniques.

Antisense Technology

[00126] Thymosin β 16 expression may also be inhibited in vivo by the use of antisense technology. Gene expression can be controlled through triple-helix formation or antisense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. An antisense nucleic acid molecule which is complementary to a nucleic acid molecule encoding thymosin β 16 can be designed based upon the isolated nucleic acid molecules encoding thymosin β 16. An antisense nucleic acid molecule can comprise a nucleotide sequence which is complementary to a coding strand of a nucleic acid, e.g. complementary to an mRNA sequence, constructed according to the rules of Watson and Crick base pairing, and can hydrogen bond to the coding strand of the nucleic acid. The antisense sequence complementary to a sequence of an mRNA can be complementary to a sequence in the coding region of the mRNA or can be complementary to a 5' or 3' untranslated region of the mRNA. Furthermore, an antisense nucleic acid can be complementary in sequence to a regulatory region of the gene encoding the mRNA, for instance a transcription initiation sequence or regulatory element. Preferably, an antisense nucleic acid complementary to a region preceding or spanning the initiation codon or in the 3' untranslated region of an mRNA is used. An antisense nucleic acid can be designed based upon the nucleotide sequence shown in SEQ ID NO:2 or 3 (thymosin β 16). A nucleic acid is designed which has a sequence complementary to a sequence of the coding or untranslated region of the shown nucleic acid. Alternatively, an antisense nucleic acid can be designed based upon sequences of the thymosin β 16 gene, which can be identified by screening a genomic DNA library with an isolated

nucleic acid of the invention. For example, the sequence of an important regulatory element can be determined by standard techniques and a sequence which is antisense to the regulatory element can be designed.

[00127] The antisense nucleic acids and oligonucleotides of the invention can be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art. The antisense nucleic acid or oligonucleotide can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids e.g. phosphorothioate derivatives and acridine substituted nucleotides can be used. Alternatively, the antisense nucleic acids and oligonucleotides can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (i.e. nucleic acid transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest). The antisense expression vector is introduced into cells in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, Reviews - Trends in Genetics, Vol. 1 (1)1986.

[00128] In addition, ribozymes can be used to inhibit in vitro expression of thymosin β 16. For example, the nucleic acids of the invention can further be used to design ribozymes which are capable of cleaving a single-stranded nucleic acid encoding a thymosin β 16 protein, such as a thymosin β 16 mRNA transcript. A catalytic RNA (ribozyme) having ribonuclease activity can be designed which has specificity for an mRNA encoding thymosin β 16 based upon the sequence of a nucleic acid of the invention (e.g., SEQ ID NO: 1). For example, a derivative of a Tetrahymena L-19 IVS RNA can be constructed in which the base sequence of the active site is complementary to the base sequence to be cleaved in a thymosin β 16-encoding mRNA. See for example Cech, et al., U.S. Pat. No. 4,987,071; Cech, et al., U.S. Pat. No. 5,116,742. Alternatively, a nucleic acid of the invention could be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See for example Bartel, D. and Szostak, J. W. Science 261:

1411-1418 (1993). RNA-mediated interference (RNAi) (Fire, et al., Nature 391: 806-811, 1998) may also be used.

RNAi Technology

[00129] RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire et al. (1998) Nature 391, 806-811). dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi involves mRNA degradation of a target gene. Results showed that RNAi is ATP-dependent yet uncoupled from mRNA translation. That is, protein synthesis is not required for RNAi in vitro. In the RNAi reaction, both strands (sense and antisense) of the dsRNA are processed to small RNA fragments or segments of from about 21 to about 23 nucleotides (nt) in length (RNAs with mobility in sequencing gels that correspond to markers that are 21-23 nt in length, optionally referred to as 21-23 nt RNA). Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA, which demonstrates that the small RNA species is generated by processing of the dsRNA and not as a product of dsRNA-targeted mRNA degradation. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21-23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21-23 nucleotide fragments from the dsRNA are guiding mRNA cleavage. Isolated RNA molecules (double-stranded; single-stranded) of from about 21 to about 23 nucleotides mediate RNAi. That is, the isolated RNAs mediate degradation of mRNA of a gene to which the mRNA corresponds (mediate degradation of mRNA that is the transcriptional product of the gene, which is also referred to as a target gene). Isolated RNA molecules specific to thymosin β 16 mRNA, which mediate RNAi, are antagonists useful in the method of the present invention. See for example U.S. Patent Application Nos: 20030153519A1; 20030167490A1; and U.S. Patent Nos: 6,506,559; 6,573,099, which are herein incorporated by reference in their entirety.

[00130] The term "pharmaceutically acceptable" refers to compounds and compositions which may be administered to mammals without undue toxicity. Exemplary pharmaceutically acceptable salts include mineral acid salts such as

hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like.

[00131] The antibodies, nucleic acids or antagonists of the invention are administered orally, topically, or by parenteral means, including subcutaneous and intramuscular injection, implantation of sustained release depots, intravenous injection, intranasal administration, and the like. Accordingly, antibodies or nucleic acids of the invention may be administered as a pharmaceutical composition comprising the antibody or nucleic acid of the invention in combination with a pharmaceutically acceptable carrier. Such compositions may be aqueous solutions, emulsions, creams, ointments, suspensions, gels, liposomal suspensions, and the like. Suitable carriers (excipients) include water, saline, Ringer's solution, dextrose solution, and solutions of ethanol, glucose, sucrose, dextran, mannose, mannitol, sorbitol, polyethylene glycol (PEG), phosphate, acetate, gelatin, collagen, Carbopol Registered TM, vegetable oils, and the like. One may additionally include suitable preservatives, stabilizers, antioxidants, antimicrobials, and buffering agents, for example, BHA, BHT, citric acid, ascorbic acid, tetracycline, and the like. Cream or ointment bases useful in formulation include lanolin, Silvadene Registered TM (Marion), Aquaphor Registered TM (Duke Laboratories), and the like. Other topical formulations include aerosols, bandages, and other wound dressings. Alternatively one may incorporate or encapsulate the compounds in a suitable polymer matrix or membrane, thus providing a sustained-release delivery device suitable for implantation near the site to be treated locally. Other devices include indwelling catheters and devices such as the Alzet Registered TM minipump. Ophthalmic preparations may be formulated using commercially available vehicles such as Sorbicare Registered TM (Allergan), Neodecadron Registered TM (Merck, Sharp & Dohme), Lacrilube Registered TM, and the like, or may employ topical preparations such as that described in U.S. Pat. No. 5,124,155, incorporated herein by reference. Further, one may provide an antagonist in solid form, especially as a lyophilized powder. Lyophilized formulations typically contain stabilizing and bulking agents, for example human serum albumin, sucrose, mannitol, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co.).

[00132] The amount of antibody, nucleic acid or inhibitor required to treat any particular disorder will of course vary depending upon the nature and severity of the

disorder, the age and condition of the subject, and other factors readily determined by one of ordinary skill in the art.

Immunotherapy

[00133] In further aspects, the present invention provides methods for using thymosin β 16 or an immunoreactive polypeptide thereof (or DNA encoding the protein or polypeptides) for immunotherapy of cancer in a patient, preferably prostate cancer. As used herein, a "patient" refers to any warm-blooded animal, preferably a human. A patient may be afflicted with a disease, or may be free of detectable disease. Accordingly, thymosin β 16 or an immunoreactive polypeptide thereof may be used to treat cancer or to inhibit the development of cancer.

[00134] In accordance with this method, the protein, polypeptide or DNA is generally present within a pharmaceutical composition and/or a vaccine. Pharmaceutical compositions may comprise the full length protein or one or more immunogenic polypeptides, and a physiologically acceptable carrier. The vaccines may comprise the full length protein or one or more immunogenic polypeptides and a non-specific immune response enhancer, such as an adjuvant, biodegradable microsphere (PLG) or a liposome (into which the polypeptide is incorporated).

[00135] Alternatively, a pharmaceutical composition or vaccine may contain DNA encoding thymosin β 16 or an immunogenic polypeptide thereof, such that the full length protein or polypeptide is generated in situ. In such pharmaceutical compositions and vaccines, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacteria and viral expression systems. Appropriate nucleic acid expression systems contain the necessary DNA sequences for expression in the patient (such as a suitable promoter). Bacterial delivery systems involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an epitope of a prostate cell antigen on its cell surface. In a preferred embodiment, the DNA may be introduced using a viral expression system (e.g., vaccinia or other pox virus, retrovirus, or adenovirus), which may involve the use of a non-pathogenic (defective), replication competent virus. Suitable systems are disclosed, for example, in Fisher-Hoch et al., PNAS 86:317-321, 1989; Flexner et al., Ann. N.Y. Acad. Sci. 569:86-103, 1989; Flexner et al., Vaccine 8:17-21, 1990; U.S. Pat. Nos. 4,603,112,

4,769,330, and 5,017,487; WO 89/01973; U.S. Pat. No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *iotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991; Kolls et al., *PNAS* 91:215-219, 1994; Kass-Eisler et al., *PNAS* 90:11498-11502, 1993; Guzman et al., *Circulation* 88:2838-2848, 1993; and Guzman et al., *Cir. Res.* 73:1202-1207, 1993. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. The DNA may also be "naked," as described, for example, in published PCT application WO 90/11092, and Ulmer et al., *Science* 259:1745-1749 (1993), reviewed by Cohen, *Science* 259:1691-1692 (1993).

[00136] Routes and frequency of administration, as well as dosage, will vary from individual to individual and may parallel those currently being used in immunotherapy of other diseases. In general, the pharmaceutical compositions and vaccines may be administered by injection (e.g., intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (e.g., by aspiration) or orally. Between 1 and 10 doses may be administered over a 3-24 week period. Preferably, 4 doses are administered, at an interval of 3 months, and booster administrations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of polypeptide or DNA that is effective to raise an immune response (cellular and/or humoral) against tumor cells, e.g., prostate tumor cells, in a treated patient. A suitable immune response is at least 10-50% above the basal (i.e. untreated) level. In general, the amount of polypeptide present in a dose (or produced in situ by the DNA in a dose) ranges from about 1 pg to about 100 mg per kg of host, typically from about 10 pg to about 1 mg, and preferably from about 100 pg to about 1 μ g. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.01 mL to about 5 mL.

[00137] Thymosin β 16 or an immunogenic polypeptide thereof can be used in cell based immunotherapies, i.e. stimulation of dendritic cells with thymosin β 16 or fusion with thymosin β 16 expressing cells. The modified dendritic cells, once injected into the patient, are a cellular vaccine, where the dendritic cells activate an immune response against the thymosin β 16 expressing cancer.

[00138] While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will vary depending on the mode of administration. For parenteral administration,

such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax and/or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and/or magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polyleptic galactide) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Pat. Nos. 4,897,268 and 5,075,109.

[00139] Any of a variety of non-specific immune response enhancers may be employed in the vaccines of this invention. For example, an adjuvant may be included. Most adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a nonspecific stimulator of immune response, such as lipid A, Bordella pertussis or Mycobacterium tuberculosis. Such adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Mich.) and Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.).

[00140] All references cited above or below are herein incorporated by reference.

[00141] The present invention is further illustrated by the following Example. This Example is provided to aid in the understanding of the invention and are not construed as a limitation thereof.

EXAMPLES

Example 1

[00142] Following the discovery of T β 15 searches NCBI expressed tag (EST) data base were performed to determine if other mammalian species possess a T β 15 ortholog. These searches uncovered a human EST (Hillier et al. 1995, unpublished, genebank accession R08518) encoding a novel β -thymosin protein, with many of the same characteristics displayed by T β 15. Using the EST data, we proceeded to clone the complete cDNA from human prostatic carcinoma LnCap cells, and have confirmed that the amino acid sequence deduced from the cDNA sequence is correct.

[00143] In Figure 1 we examined the level of endogenous β -thymosin proteins (T β 4, T β 15, T β 16) in the LnCaP human prostate cancer cell line, originally derived from a lymph node metastasis, and in low or high metastatic variants of the Dunning

rat prostatic carcinoma cell line. Western blot analysis revealed that LnCaP cells produce thymosin T β 16 but not thymosin T β 4 or T β 15. In contrast the high metastatic variant of the Dunning rat prostatic carcinoma contains T β 4 and T β 15 but not T β 16, whereas the T β 4 is the only form of β thymosin found in the nonmetastatic variant. This is the first time the endogenous T β 16 protein has been detected in any cell type.

Example 2

Immunohistochemistry – Summary of TB16 expression in human prostatic carcinoma.

[00144] The results (Fig. 2) show a general correlation between thymosin β 16 staining and the Gleason score. For instance, a higher percentage of high-grade tumors exhibited partial or positive staining (90%, Gleason score 8 – 10) than did medium-grade tumors (59%, Gleason score 6 – 7), or low grade tumors (Gleason score 2 – 5). It is important to note that medium-grade prostatic carcinomas (Gleason scores 6 – 7) are found in all three categories. Presently, Gleason scores of 6 to 7 have no predictive value regarding distant failure in patients with clinically localized prostate cancer. As such, thymosin β 16 is an independent predictor of distant failure for patients with moderately differentiated prostate carcinomas. By contrast, T β 16 staining was absent in nine specimens of benign prostatic hyperplasia (BPH). In the one case of BPH where a T β 16 signal was observed, staining was weak and limited to a single prostate gland. Moreover, prostate specific antigen (PSA) is considered the gold standard of prostate cancer diagnosis, yet PSA is highly expressed in BPH tissues.

Example 3

β -thymosin RNA expression profile in low and high metastatic cell lines.

[00145] Northern blot analysis of β -thymosin RNA expression in low and high metastatic cell lines was performed. Figure 3 demonstrates that a changes in metastatic phenotype alters the β -thymosin expression profile in tumor cells. β -thymosin isoforms T β 15 and T β 16 are found primarily in highly metastatic cells including those that originate from prostate or breast carcinomas. For instance T β 16 levels are higher in subclones of LnCaP prostate carcinoma cells and MDA-MB-435 breast carcinoma cells selected for increased metastatic phenotype. Like T β 15 or T β 16, thymosin β 10 also rose with increasing metastatic phenotype in many cell types

(e.g. Dunning, PC3M, MDA-MB), but decreased in others (e.g. LnCaP). By contrast, levels of the thymosin β 4 isoform tend to be highest only in cells that produce fewer metastases (e.g. PC3M-L & MDA-MB-435-L) than parental cell lines or the highly metastatic variants.

[00146] In Figure 3, DU stands for DU 145 which is a prostate cancer cell line from a lesion in the brain of a patient with metastatic carcinoma of the prostate. PC3M is a derivative of the PC3 cell line, which is a prostate cancer cell line isolated from a bone metastasis of a grade IV prostatic adenocarcinoma from a 62-year-old male Caucasian. PC3M was reisolated as a cell line from a tumor metastasis which formed in a SCID mouse. BxPC-3 (or Bx) is a cell line isolated from a pancreatic carcinoma.

Example 4

T β 16 is expressed at low levels in normal human adult tissues and expression increases in a variety of tumors..

[00147] T β 16 mRNA expression in normal human adult and fetal tissues was monitored (Figure 4). The results show that T β 16 mRNA is absent or expressed at low levels in normal adult tissues compared to the endogenous levels of β -actin mRNA which encodes the primary β -thymosin binding partner (Fig 4A vs. Fig. 4C). Moderate levels of T β 15 expression were observed in normal tissues from brain and fetal lung. Like β -actin, thymosin β 16 belongs to a multigene family with multiple pseudogenes and the signal from the genomic DNA control spots reflects hybridization to the genomic alleles. However, these hybridization control spots in the T β 16 blot yielded much stronger signals than any normal tissue, whereas tissues accounted for the vast majority of signal on the β -actin blot. Even in tissues with detectable levels of T β 16 mRNA (e.g. prostate, ovary), when taken together this data indicates that T β 16 is expressed at low levels in normal human adult tissues.

[00148] The levels of T β 16 assessed in a variety of tumors. Figure 5 shows that increased expression of T β 16 was found in Prostate cancer, Lung carcinoma, Breast carcinoma, Thyroid carcinoma, Brain cancers (cerebellum, medulloblastoma, astrocytoma, ependymoma, glioblastoma), Eye cancer (retinoblastoma), Muscle (rhabdomyosarcoma), Pancreatic cancer, Lymphoma (T or B cell), Stomach cancer, and Ovarian carcinoma, as determined by either (a) Antibody staining (b) Express

Sequence Tag analysis (c) Serial Analysis of Gene Expression (SAGE) (d) Gene Chip Array (e) or Northern Blot analysis.

Example 5

Expression of β -thymosin isoforms T β 4 and T β 16 in various human tissues by Northern Blot analysis.

[00149] A multiple-tissue Northern blot of normal human tissue (Clontech, PT1200-1) was sequentially hybridized with T β 16 and T β 4 32 P labeled DNA probes (Figure 6). β -actin is shown as a loading control. Thymosin β 16 mRNA could not be detected in any of the normal tissues present on the multiple-tissue northern blot (Clontech, CA). In contrast, T β 4 a ubiquitous β -thymosin isoform, was highly expressed in all tissues, with the exception of muscle tissues which contained moderate amounts of the T β 4 mRNA. Similar quantities of the β -actin loading control were observed in all tissues, although other highly conserved actin isoforms are probably responsible for the signal in muscle tissues.

[00150] The references cited throughout the specification are incorporated herein by reference. The present invention has been described with reference to specific embodiments. However, this application is intended to cover those changes and substitutions that may be made by those skilled in the art, without departing from the spirit and the scope of the appended claims.

[00151] TB16 protein

MSDKPDLSEVEKFDRSKLKKTNTEEKNTLPSKETIQQEKECVQTS (SEQ ID No:1)

[00152] TB16 RNA allele1

GCGGGAAGGCTAACCTGGTGC GGAGCCAGCCTGGGTCTCAGCCCCGCGTA
CGGCCTTTCACGAGTCTTCAAGCCTTCAGGCTTCTTCTAGTCAAGATGAG
TGATAAACCACTTGTTCGGAAGTGGAGAAGTTTGACAGGTCAAACTGA
AGAAAATAATACTGAAGAAAAAATACTCTTCCCTCAAAGGAACTATC
CAGCAGGAGAAAGAGTGTGTTCAAACATCATAAAATGGGGATCTCCTCCA
AAGAGCAGATTTTCAGCATTGCCTGACAGTCTTGGTTTTAGGCTTGTTTTT
TGTAACCTGTGTGTTTGTAGAGATTTTCAGACATCTTCTGATTTCTTCTCAC
CTATATTCCCTGGTTAAGAGGTCAGGGGTAGTGAATGTTTCCTTAAGTTCC
TTTTTAACTTCCCATTGGTATGTAAATTCCAAATGGCAGATGCTGTCAAT
AACCTTGCCATGGATGACCTTTGTGTAGGTAGTTCCTTGACCTCATGCAGG
ATAAGCCAATTTTAACTTTCTACAATGGGTGCCTCAATAGTTTCATAATCT
TCATGAAGTTGCATCCTTTGGCAGCTTCTTACAGTTTATTTTCACTTCCAAT
GTAGCAATAAAATAATAAATATAATCGTT (SEQ ID NO:2)

[00153] TB16 RNA allele2

GCGGGAACGCTAACCTGGTCCGGAGCGAGTCTGGGTCTCAGCCCCGCGAA
CAGCCTTTCACGAGTCTTCAAGCTTTCAGGCTATCTTCTAGTCAAGATGAG
TGATAAGCCAGACTTGTTCGGAAGTGGAGAAGTTTGACAGGTCAAACTGA
AGAAAATAATACTGAAGAAAAAATACTCTTCCCTCAAAGGAACTATC
CAGCAAGAGAAAGAGTGTGTTCAAACATCATAAAATGGGGATCGCCTCCC
AACAGCAGATTTTCGACATTACCTGAGAGTCTTGATTTTAGGCTTGTTTTT
GTAAACCCATGTGTTTGTAGAGATTTTAGGCGTCTTCGGATATCTTCTCAC
CTATGTTCCCTGGCTAAGAAGTCAGAGGTAGCCAATGTTTCCTTAAATTCA
TTTTTAACTTACCATTGGTGCATATGTTCCAGATGGCAGATGCTGTCAAT
AATCTCACCATTGATGACCTTTGTGTATGTAGTTCTTGATCCTATACTGG
ATAAGCCTGTTTTAACCTGCTATGATGGGTGCTTCCATTGCTTCATAATCTT
CATGAAGTTGCATGCTTTTGCAGCTTTTCACAGTTTATTTGCATTTCTAATG
TAGTAATAAAGTAACCAATATAATCATT (SEQ ID NO:3)